Behavioural endophenotypes in mice lacking the auxiliary GABAB receptor subunit KCTD16

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Abstract: Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and is implicated in the pathophysiology of a number of neuropsychiatric disorders. The GABAB receptors are G-protein coupled receptors consisting of principle subunits and auxiliary potassium channel tetramerization domain (KCTD) subunits. The KCTD subunits 8, 12, 12b and 16 are cytosolic proteins that determine the kinetics of the GABAB receptor response. Previously, we demonstrated that Kctd12 null mutant mice (Kctd12(-/-)) exhibit increased auditory fear learning and that Kctd12(+/-) mice show altered circadian activity, as well as increased intrinsic excitability in hippocampal pyramidal neurons. KCTD16 has been demonstrated to influence neuronal excitability by regulating GABAB receptor-mediated gating of postsynaptic ion channels. In the present study we investigated for behavioural endophenotypes in Kctd16(-/-) and Kctd16(+/-) mice. Compared with wild-type (WT) littermates, auditory and contextual fear conditioning were normal in both Kctd16(-/-) and Kctd16(+/-) mice. When fear memory was tested on the following day, Kctd16(-/-) mice exhibited less extinction of auditory fear memory relative to WT and Kctd16(+/-) mice, as well as more contextual fear memory relative to WT and, in particular, Kctd16(+/-) mice. Relative to WT, both Kctd16(+/-) and Kctd16(-/-) mice exhibited normal circadian activity. This study adds to the evidence that auxillary KCTD subunits of GABAB receptors contribute to the regulation of behaviours that could constitute endophenotypes for hyper-reactivity to aversive stimuli in neuropsychiatric disorders.

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Behavioural endophenotypes in mice lacking the auxiliary $GABA_B$ receptor subunit KCTD16

Running title: Behavioural endophenotypes in *Kctd16* knock-out mice

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Abstract

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain and is implicated in the pathophysiology of a number of neuropsychiatric disorders. The $GABA_B$ receptors are G-protein coupled receptors consisting of principle subunits and auxiliary potassium channel tetramerization domain (KCTD) subunits. The KCTD subunits 8, 12, 12b and 16 are cytosolic proteins that determine the kinetics of the $GABA_B$ receptor response. Previously, we demonstrated that *Kctd12* null mutant mice (*Kctd12*$^{-/-}$) exhibit increased auditory fear learning and that *Kctd12*$^{+/-}$ mice show altered circadian activity, as well as increased intrinsic excitability in hippocampal pyramidal neurons. KCTD16 has been demonstrated to influence neuronal excitability by regulating $GABA_B$ receptor-mediated gating of postsynaptic ion channels. In the present study we investigated for behavioural endophenotypes in *Kctd16*$^{-/-}$ and *Kctd16*$^{+/-}$ mice. Compared with wild-type (WT) littermates, auditory and contextual fear conditioning were normal in both *Kctd16*$^{-/-}$ and *Kctd16*$^{+/-}$ mice. When fear memory was tested on the following day, *Kctd16*$^{-/-}$ mice exhibited less extinction of auditory fear memory relative to WT and *Kctd16*$^{+/-}$ mice, as well as more contextual fear memory relative to WT and, in particular, *Kctd16*$^{+/-}$ mice. Relative to WT, both *Kctd16*$^{+/-}$ and *Kctd16*$^{-/-}$ mice exhibited normal circadian activity. This study adds to the evidence that auxillary KCTD subunits of $GABA_B$ receptors contribute to the regulation of behaviours that could constitute endophenotypes for hyper-reactivity to aversive stimuli in neuropsychiatric disorders.

Keywords*:* Gamma-aminobutyric acid; $GABA_B$; KCTD16; fear learning, memory, extinction; neuropsychiatric disorders

Abbreviations:

BLA basolateral amygdala; Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KCTD potassium channel tetramerization domain; Nonidet non-ionic, non-denaturing detergent; NP nonyl phenoxypolyethoxylethanol

1. Introduction

The $GABA_B$ receptors are G-protein coupled receptors for gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) [1]. They are expressed by almost all neurons and glia in the CNS and are major regulators of synaptic transmission and neuronal excitability, thereby modulating emotion and cognition [2, 3]. The $GABA_B$ receptors are hetero-multimers composed of principal and auxiliary subunits. The principal subunits $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ form fully functional $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors that localize to pre- and postsynaptic sites, respectively [4, 5]. These receptors can associate with the cytosolic auxiliary potassium channel tetramerization domain (KCTD) subunits KCTD8, KCTD12, KCTD12b or KCTD16. The KCTD proteins bind constitutively to $GABA_{B2}$ to form receptor subtypes that are structurally and functionally distinct [6]. The KCTDs simultaneously bind to the $GABA_B$ receptor and the G-protein, and thereby influence the kinetics of the receptor response via stabilization of the G-protein at the receptor and direct effects on G-protein signaling [7].

Postmortem studies have identified decreased $GABA_B$ receptor density in patients with schizophrenia [8, 9] and decreased $GABA_{B1}$ and $GABA_{B2}$ protein expression in patients with schizophrenia, bipolar disorder or major depressive disorder (MDD) [10]. Studies in laboratory animals reported increases in $GABA_B$ receptor density and mRNA expression, or function in response to administration of antidepressants [11-14]. In addition, $GABA_B$ receptor antagonists showed anxiolytic and antidepressant-like activity in a number of animal models [15-19]. Accordingly, $GABA_B$ receptors have been proposed as a potential treatment target for these major neuropsychiatric disorders as well as for anxiety disorders and addiction [20-23]. The KCTD proteins are also associated with neuropsychiatric disorders. KCTD12 has been most studied: a genome-wide association study of bipolar disorder I patients identified an association with a single nucleotide polymorphism in a region that includes *KCTD12* [24]; a translational microarray study identified *KCTD12*/*Kctd12* up-regulation in the amygdala of MDD patients and stressed mice relative to their respective control groups [25]. *KCTD16* was identified as a candidate gene for a congenital partial epilepsy syndrome [26]. A *KCTD8* polymorphism was associated with brain size in female adolescents, with its proposed role being KCTD8 modulation of the adverse effects of prenatal exposure to maternal cigarette smoking on cortical development [27].

The availability of mice mutant for the $GABA_B$ receptor principal subunits has allowed for elucidation of their behavioural roles. Thus, genetic ablation of either $GABA_{B1}$ or $GABA_{B2}$, which results in mice lacking functional $GABA_B$ receptors, induced antidepressant-like behavioural effects [15, 28], cognitive impairments [29-31] and alterations in the circadian organization of sleep [32]. The

contribution of receptor subtypes to physiological and behavioral $GABA_B$ functions was assessed in mice lacking specific $GABA_{B1}$ subunit isoforms [3-5, 33, 34]. In this respect, the emotional processing and learning and memory of aversive stimuli have been studied using Pavlovian fear conditioning: an initially neutral stimulus, either a discrete event such as a tone (conditioned stimulus, CS) or the general context, is paired with an aversive unconditioned stimulus (US), typically a foot shock [35]. Fear learning, memory and memory extinction learning are then assessed by measuring freezing to the CS or context [35]. In $GABA_{B1a}^{-/-}$ mice i.e. mice lacking the $GABA_{B1}$ subunit that localizes to presynaptic sites, tone CS fear learning and memory were normal relative to wild-type (WT), whilst there was an endophenotype of increased generalized fear to a neutral tone CS and a neutral context. In $GABA_{B1b}^{-/-}$ mice i.e. mice lacking the $GABA_{B1}$ subunit that localizes to postsynaptic sites, in contrast, fear learning was completely impaired. These critical and distinct roles of specific $GABA_{B1}$ subunit isoforms reflect their major involvement in pre- and postsynaptic modulation of the long-term potentiation induced by thalamo-cortical inputs to the amygdala [36]. In conditioned taste aversion (CTA), another form of aversive learning and memory involving pairing of normally rewarding sweet taste with malaise induced by lithium chloride injection, $GABA_{B1a}^{-/-}$ mice failed to acquire CTA. $GABA_{B1b}^{-/-}$ mice acquired CTA to a similar extent to WT; however, they differed from WT in terms of a robust deficit in extinction learning across repeated exposure to the sweet taste in the absence of malaise [37]. $GABA_{B1}$ subunit-specific mutant mice have also been studied in terms of circadian activity endophenotypes: $GABA_{B1a}^{-/-}$ mice displayed higher inactive phase activity than WT, whereas $GABA_{B1b}^{-/-}$ mice showed active phase hyperactivity relative to WT [38]. Recently, behavioural studies have been conducted with mice lacking the gene for specific auxillary KCTD subunits. We demonstrated that *Kctd12*$^{-/-}$ mice have an endophenotype of increased fear learning and that *Kctd12*$^{+/-}$ mice have an endophenotype of hyperactivity during the inactive phase of the circadian cycle, relative to WT. Furthermore, *in vitro* electrophysiological recordings from the hippocampus, a region of high *Kctd12* expression, revealed an increased intrinsic excitability of pyramidal neurons in *Kctd12*$^{-/-}$ and *Kctd12*$^{+/-}$ mice relative to WT mice [39].

The auxillary subunit KCTD16 is highly expressed in a number of brain regions including amygdala and hippocampus [40]. It has been demonstrated to regulate the $GABA_B$ receptor-mediated gating of postsynaptic ion channels [6, 41], which influences neuronal excitability, oscillatory network activity and cognitive functions [42-45]. Here we report on experiments conducted with *Kctd16*$^{-/-}$, *Kctd16*$^{+/-}$ and WT mice aimed at assessing whether KCTD16 regulates behavioural states that could constitute risk factors (endophenotypes) for neuropsychiatric disorders in terms of fear learning and memory and circadian activity.

2. Material and methods

2.1. Animals and housing

Male and female *Kctd16*$^{+/-}$ mice on a BALB/c background were paired to generate offspring for each genotype i.e. WT, *Kctd16*$^{+/-}$ and *Kctd16*$^{-/-}$. Mice were weaned at age 3-4 weeks and ear tissue punches were obtained at age 5-7 weeks for genotyping. All animals were caged as littermate pairs-trios in an individually-ventilated cage system (IVC), and were maintained on a reversed 12:12 h light-dark cycle (white light off at 07:00 h). Temperature was set at 20-22 °C and humidity at 50-60 %. Food (Complete pellet, Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were both available continuously and *ad libitum*. Mice were handled on three days prior to each experiment, and all tests were conducted between 11:00 h and 16:00 h. Each behavioural experiment was performed with naive males aged 8-16 weeks, derived from at least five different litters. There was no effect of genotype on body weight: WT 24.1 ± 1.4 g, *Kctd16*$^{+/-}$ 23.8 ± 1.0 g, *Kctd16*$^{-/-}$ 27.1 ± 1.6 g (p=0.46). All procedures were conducted under permits (170/2012, 1897/2015) for animal experimentation issued by the Veterinary Office of Zurich or Basel-Stadt, Switzerland.

2.2. Genotyping

Mice were genotyped by PCR using genomic DNA extracted from ear tissue. To distinguish between the WT and knock-out *Kctd16* alleles, 34 cycles of PCR with primers P1 (5'-TTT GCC CTT GCC TGC AGG T-3'), P2 (5'-ACC GAG AGG ATG CTG AGT C-3') and P3 (5'- AGC CAA GCT AGC GAA GTT CC-3') were employed (annealing at 53 °C for 30 s and extension at 72 °C for 1 min), generating fragments of 245 bp for the WT allele and 386 bp for the knock-out allele.

2.3. Western Blotting

Brain membranes were prepared as previously described [29]. Briefly, mouse brains were hemi-dissected and homogenized on ice using a Dounce homogenizer in 10 volumes of buffer containing 4 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 0.32 M sucrose. Homogenates were cleared by centrifugation at 1000 g for 10 min, and the supernatants again centrifuged at 40000 g for 30 min. The pellets containing the brain membranes were solubilized by rocking the tubes for 3 h at 4 °C in NP-40 buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1mM EDTA, 0.5 % (v/v) Nonidet P-40) containing Complete EDTA-free Protease Inhibitor Cocktail (Roche, 11 836 170 001). Solubilized brain membranes were then cleared by centrifugation at 16000 g for 10 min at 4 °C and resolved on standard 12 % SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, IPVH00010). Membranes were then blocked in 5 % BSA in PBS containing 0.1 % Tween-20 (PBST) and probed with the following primary antibodies: Rabbit anti-KCTD8, rabbit anti-KCTD12, rabbit anti-KCTD16 (all at 1:2500) [40], rabbit-anti KCTD12b (N-terminal peptide

MAMPEKSSDVKPTEEC, at 1:1000) overnight at 4 °C and mouse anti-tubulin (BD Pharmingen, Clone 5H1, 1:2500) for 1 h at room temperature. Incubation with peroxidase-coupled secondary antibodies (Amersham Biosciences, NA931V and NA9340V, 1:10000) was for 1 h at room temperature. Primary antibody incubation was in 5 % BSA in PBST and secondary antibody incubation was in 5 % non-fat dry milk in PBST. Following antibody incubations, membranes were washed 4 times for 10 min in PBST. Western blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and captured under non-saturating conditions with a Fusion FX image acquisition system (Vilber Lourmat).

2.4. Open field test

Naive mice, eight per genotype, were habituated to the remote experimental room for 24 h prior to testing. The open field consisted of a black Plexiglas arena measuring 50 (L) x 50 (W) x 40 (H) cm [39]. Lighting was set to 15 lux at the arena centre. The arena was cleaned with 70 % ethanol prior to each test. The mouse was placed in the centre of the arena to begin the 30 min open field test. Mouse location and movement were monitored using a video-tracking system (VideoMot 2, Version 5.76, TSE Systems GmbH, Bad Homburg, Germany). The arena floor was divided virtually into centre (30 x 30 cm) and periphery. The test measures, namely percent time spent in and distance moved per region, were analyzed in 5-min bins.

2.5. Fear learning and memory

Fear conditioning experiments were conducted using an automated apparatus (MultiConditioning System, TSE Systems GmbH) as described in detail elsewhere [46, 47]. A dark-walled Plexiglas arena (30 x 30 x 24 cm) was placed on an electrified grid floor measuring 30 x 30 cm and comprising 29 stainless steel rods. A black metal waste tray was placed under the grid floor. Infrared light-beam sensors allowed for measurement of movement in three dimensions. Each such conditioning chamber, four in total, was placed in an attenuation chamber equipped with a ventilation fan and house lights set to give illumination of 8 lux. An electroshock of 2 s duration and 0.2 mA current constituted the aversive unconditioned stimulus (US). The discrete conditioned stimulus (CS) consisted of a tone (5 kHz, 85 dB) that was presented for 20 s with the final 2 s contiguous with the US. Mice were tested in either a CS-US or Context-US fear learning and memory paradigm.

2.5.1. CS-US fear conditioning

This experiment was conducted on three consecutive days, with eight naive mice per genotype. *Day 1, baseline:* Mice were placed in the arena (context) for 15 min without CS or US. *Day 2, conditioning:* Following 120 s adaptation, mice were exposed to six CS-US pairings with an inter-trial interval (ITI)

of 120 s. *Day 3, fear expression test:* For the CS fear expression test mice were placed in a modified context: A dark Plexiglas divider containing a door (3.5 (W) x 10 (H) cm) was placed across the centre of the arena to form two compartments and the waste tray below the grid floor was changed from black to white. After an adaptation time of 180 s mice were exposed to 18 x 30-s CS separated by 90-s ITI.

2.5.2. Context-US fear conditioning

This experiment was conducted on two consecutive days, with eight naive mice per genotype. *Day 1, conditioning:* Mice were given 180 s adaptation followed by 6 x 2-s US with an ITI of 138-s. *Day 2, fear expression test:* Mice were tested in the same context for 18 x 60-s trials.

Freezing was defined as the complete absence of movement detection for at least 2 s, and periods of freezing were expressed as percent time spent freezing per CS or ITI. Mean % time freezing per mouse was calculated for each 2-3 consecutive CSs or ITIs, depending on experiment and phase. Distance moved during USs was also measured and used to assess effects of genotype on pain reactivity.

2.6. Circadian activity

Circadian activity of individual mice was studied using IntelliCage (TSE), an automated system for monitoring home-cage activity [48]. Each mouse is fitted with a subcutaneous transponder that records: visits to the operant devices located in the cage corners; operant nosepokes into a sensor (light-barrier) at the two doors per corner that open to allow access to drinking bottles; and the number of drinking licks measured via electrical contact of the tongue with the tip of the water bottle cap. Each IntelliCage of 55 (W) x 38 (L) x 20 (H) was divided at the centre by a solid PVC barrier to give two independent cages each containing two such operant corner units. Each of the four IntelliCages used was placed in its attenuation chamber with a reversed 12:12 h dark-light cycle and individual ventilation. Naive mice, 6 per genotype, were studied in littermate pairs, each pair in one cage-half. For the first five days mice were habituated to the cage with operant doors open, followed by closing the doors and operant training with one nosepoke required to open a door for 10 s, for the next five days. Data were then collected for 15 days, with output parameters of total visits, nosepokes and licks. Behaviour was analyzed continuously across 15 days with each day divided into its 12-h dark phase and 12-h light phase.

2.7. Statistical analysis

Behavioural data were analyzed using SPSS (version 19, SPSS Inc., Chicago IL, USA). ANOVA was conducted with a between-subject factor of genotype and, depending on behavioural test, a within-subject factor of trial. Linear regression analysis was conducted using GraphPad Prism (Version 6.07). Statistical significance was set at $p<0.05$. Data are presented as mean ± standard error (SEM).

3. Results

3.1. Generation of Kctd16 deficient mice and effects on protein expression

Kctd16$^{-/-}$ mice were generated by targeted mutagenesis of the *Kctd16* gene in BALB/c embryonic stem (ES) cells (Fig. 1A). Correctly targeted ES cells were injected into C57BL/6 blastocysts and chimeric males were crossed with BALB/c females. This resulted in a F1 generation of BALB/c *Kctd16*$^{+/-}$ mice heterozygous for the *Kctd16* knock-out allele. In all the studies described below we used *Kdtd16*$^{+/+}$, *Kctd16*$^{+/-}$ and *Kctd16*$^{-/-}$ mice derived from *Kctd16*$^{+/-}$ x *Kctd16*$^{+/-}$ breeding pairs. *Kctd16*$^{+/-}$ mice expressed 47 % of the KCTD16 protein of WT mice ($p<0.0001$) in whole brain lysates (N=4/group) whilst KCTD16 protein was undetectable in *Kctd16*$^{-/-}$ mice. There were no compensatory alterations in protein levels of $GABA_{B1a,b}$, $GABA_{B2}$, KCTD8, KCTD12 or KCTD12b (Fig. 1B).

[Figure 1 about here]

3.2. Open field test

In the open field test, total distance moved was used as an index of activity, and distance moved in the relatively exposed centre as a measure of anxiety i.e. less distance moved in centre equates to greater anxiety [39]. For the total distance moved, there was a main effect of 5-min time-interval ($F(5, 75)=4.610$, $p<0.001$), with mice exhibiting a consistent reduction in activity across the 30-min session, as expected for this test. There was no effect of genotype (main or interaction, $p\geq0.97$). For the distance moved in centre, there was also no effect of genotype ($p\geq0.64$).

3.3. Fear learning and memory

3.3.1. CS-US fear conditioning

On day 1, mice were habituated to the novel context (fear conditioning arena) without electroshock. There was no effect of genotype on baseline % time spent freezing, which was consistently low: WT 5.7 ± 1.4 %, *Kctd16*$^{+/-}$ 3.0 ± 0.7 %, *Kctd16*$^{-/-}$ 3.4 ± 0.5 % ($p=0.11$). On day 2, CS-US conditioning (Fig. 2A), % time spent freezing during the CS of CS-US trials was measured. There was a main effect of CS-US trial ($F(2, 42)=69.276$, $p<0.001$) indicating the learning of CS fear across successive CS-US pairings. CS-US learning was similar across the different genotypes ($p\geq0.48$) (Fig. 2A). There was also no effect

of genotype on US reactivity, measured as distance moved (in arbitrary units) during 2-s footshocks: WT 901 ± 119, Kctd16$^{+/-}$ 849 ± 103, Kctd16$^{-/-}$ 689 ± 100 (session mean ± SEM, p=0.26). On day 3, fear expression test, the memory of CS fear was measured (Fig. 2B). There was a main effect of CS trial on % time freezing ($F(5, 105)=5.302$, $p<0.001$) indicating a decrease in fear memory across successive exposures to the CS in the absence of the US. This occurred in the absence of either a main effect of genotype or a genotype x CS trial interaction ($p\geq0.44$) (Fig. 2B). Nonetheless, the mean freezing profiles for the different genotypes suggested a reduced attenuation of freezing in *Kctd16*$^{-/-}$ mice relative to the other genotypes. This difference was supported statistically by the significant linear regression of % time freezing against CS trial in WT mice ($p<0.03$) and *Kctd16*$^{+/-}$ mice ($p<0.03$), and the absence of such regression in *Kctd16*$^{-/-}$ mice ($p=0.48$) (Fig. 2C).

3.3.2. Context-US fear conditioning

On day 1, conditioning, naive mice were exposed repeatedly to the aversive US in the novel context. There was a main effect of US trial ($F(2, 42)=79.606$, $p<0.001$) indicating the learning of contextual fear across successive US presentations. Context-US learning was similar across the different genotypes ($p\geq0.26$) (Fig. 2D). On day 2, fear expression test, the memory of contextual fear was measured (Fig. 2E). There was a main effect of context trial ($F(5, 105)=5.478$ $p<0.001$) indicating a decrease in fear memory across successive 60-s trials of exposure to the context in the absence of the US. There was also a main effect of genotype ($F(2, 21)=5.047$, $p=0.016$): *Kctd16*$^{-/-}$mice spent more time freezing than did *Kctd16*$^{+/-}$mice ($p=0.005$) with WT mice exhibiting intermediate freezing (*Kctd16*$^{-/-}$ vs WT $p=0.20$). *Kctd16*$^{+/-}$ mice showed a borderline non-significant decrease in freezing compared to WT mice ($p=0.08$) (Fig. 2E).

[Figure 2 about here]

3.4. Circadian activity

As expected, activity in IntelliCage was greater during the 12-h dark periods than during the 12-h light periods across the 15-day period. This was the case for each genotype, and there was no genotype effect on any measure. For example, there were more corner visits during the dark period (Fig. 3A) versus light period (Fig. 3D) (main effect of period: $F(1, 435)=1232.914$, $p<0.001$) in the absence of an effect of genotype ($p\geq0.84$). Also when the dark and light periods were analyzed separately, there were no effects of genotype: dark period, visits ($p\geq0.84$, Fig. 3A), nosepokes ($p\geq0.82$, Fig. 3B), water licks ($p\geq0.89$; Fig. 3C); light period, visits ($p\geq0.14$, Fig. 3D), nosepokes ($p\geq0.33$, Fig. 3E), water licks ($p\geq0.23$, Fig. 3F).

[Figure 3 about here]

4. Discussion

In the present study we investigated for behavioural phenotypes in adult male BALB/c mice with either homo- or heterozygous ablation of *Kctd16*, the gene encoding one of the auxiliary subunits of $GABA_B$ receptors. Relative to WT littermates, *Kctd16*$^{+/-}$ mice exhibited the expected 50 % reduction in KCTD16 protein expression in the CNS. There was no evidence for compensatory CNS changes in expression of the principal subunits $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ or the other auxiliary subunits KCTD8, 12 and 12b, neither in *Kctd16*$^{-/-}$ nor in *Kctd16*$^{+/-}$ mice. We cannot rule out that alterations in other receptor systems contribute to the observed behavioral phenotypes. For example, reduced $GABA_B$ receptor numbers and other changes in the GABAergic system were observed in the hippocampus of mGlu7-deficient mice [49]. However, given that *KCTD16* polymorphism is associated with brain disorder (e.g. [26]), our animal models with a constitutive *Kctd16* ablation (homo- or heterozygous) are relevant to human disease. Fear conditioned freezing was used to study KCTD16 effects on aversive learning and memory. In tone CS-US fear conditioning, there was no effect of genotype on emotional learning of the CS-US association; there was some evidence for reduced extinction of the CS fear memory in *Kctd16*$^{-/-}$ mice relative to WT and *Kctd16*$^{+/-}$. In context-US fear conditioning, there was again no effect of genotype on emotional learning; however, context fear memory was increased in *Kctd16*$^{-/-}$ mice relative to *Kctd16*$^{+/-}$ mice with WT intermediate. There were no effects of KCTD16 reduction/depletion on circadian activity. These findings add to the evidence that both auxillary KCTD subunits and principal $GABA_B$ subunits make distinct contributions to the overall regulation of aversive learning and memory processes by $GABA_B$ receptors.

The evidence for an absence of KCTD16 involvement in regulation of CS fear acquisition is in contrast to that obtained for KCTD12; *Kctd12*$^{-/-}$ mice exhibited increased CS fear acquisition, suggesting that this subunit modulates the extent to which $GABA_B$ contributes to CS-US association learning [39]. The evidence for mild impairment of extinction of aversive CS memory in *Kctd16*$^{-/-}$ mice is the first evidence for a contribution of the $GABA_B$ receptor to extinction in the rodent CS fear conditioning paradigm. Extinction constitutes a net reduction in the acquired association between two events following exposure to the discontinuation of that association. It is proposed to be an active process involving formation of a new, inhibitory memory [50]. Extinction of CS fear memory is associated with increased glutamate signaling from the infralimbic cortex onto amygdala and increased long-term potentiation input onto local GABAergic inhibitory circuits within the amygdala [51-53]. Studies in mice with partial or complete ablation of $GABA_B$ receptor subunits did not identify any effects on

extinction of fear memory [36]. However, in conditioned taste aversion (CTA), $GABA_{B1b}^{-/-}$ mice showed a marked and chronic deficit in CS (sweet taste associated with malaise) extinction learning [37]. In addition to the reduced aversive-CS extinction endophenotype, *Kctd16*$^{-/-}$ mice exhibited increased aversive-context memory relative to *Kctd16*$^{+/-}$ mice, with WT mice exhibiting an intermediate level of fear memory. Rather than being a deficit in extinction, the high level of context fear memory was evident throughout the expression test in *Kctd16*$^{-/-}$ mice, suggesting that the lack of KCTD16 increased memory consolidation. Context-US learning and memory occur primarily in the hippocampus, dependent on bidirectional signaling with the basolateral amygdala (BLA) [54, 55]. In the adult mouse brain, KCTD16 is expressed in the BLA and the hippocampal formation [40]. Marked aversive memories that are resistant to extinction are particularly relevant to trauma- and stress-related disorders, perhaps most notably posttraumatic stress disorder [56]. A gene-environment model approach, for example applying environmental stressors to *Kctd16*$^{-/-}$ mice, would be relevant to explore this further.

The auxillary subunit KCTD16, which as noted above is highly expressed in a number of brain regions including amygdala and hippocampus [40], regulates the $GABA_B$ receptors with respect to their gating of postsynaptic ion channels, such as hyperpolarization-activated cyclic nucleotide gated (HCN) channels [6, 41]. As such, it is well placed to influence $GABA_B$ modulation of neuronal excitability, oscillatory network activity, and cognitive functions such as learning and memory [42-45]. The different behavioural phenotypes that can be attributed to KCTD16 (present study) and KCTD12 [39] coincide with their distinct domain structures and to their being constituents of distinct receptor complexes [41, 57]. As a group and individually, therefore, evidence is emerging that auxillary KCTD subunits of $GABA_B$ receptors are of regulatory relevance to specific learning and memory processes that are important in adaptive behaviour and that also underlie important psychopathologies in neuropsychiatric disorders.

Conflict of interests

The authors declare no conflict of interest.

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Figure captions

Figure 1. **(A)** Generation of *Kctd16*-deficient mice. The first exon (E1) of the *Kctd16* gene was replaced with an FRT-flanked neomycin resistance cassette (TK-neo) by homologous recombination in embryonic stem (ES) cells. In the targeting vector the neomycin cassette was flanked by genomic sequences homologous to the targeting area in the *Kctd16* gene (long and short homology arms). The PCR primers used for genotyping are indicated by arrowheads (P1, P2 and P3). The sizes of the amplified PCR products are indicated in base pairs (bp). **(B)** Western blot analysis of KCTD16 protein expression in whole brain lysates. *Kctd16*$^{+/-}$ mice express 47% of KCTD16 compared to WT, and normal protein levels of $GABA_{B1a,b}$ (GB1a,b), $GABA_{B2}$ (GB2), KCTD8, KCTD12 or KCTD12b. Asterisk indicates cross-reacting bands.

Figure 2. Effects of *Kctd16* genotype on tone CS-US (**A** - **C**) and Context-US (**D** and **E**) fear learning and memory, measured as percent time spent freezing. CS-US conditioning: **(A)** CS learning. **(B)** CS memory expression. **(C)** Relationship between % time freezing and CS trial as estimated by linear least-means squares regression. Solid lines are for the regression equation for each genotype: WT $F(1,4)=12.45$, $p<0.03$; HET $F(1,4)=11.76$, $p<0.03$, KO $F(1,4)<1$, $p=0.48$. Context-US conditioning: **(D)** Context learning. **(E)** Context memory expression. Data are presented as mean ± SEM (N=8 mice per genotype per experiment). CS: conditioned stimulus; US: unconditioned stimulus; WT: wild type.

Figure 3. Effects of *Kctd16* genotype and time period on activity and operant drinking behaviour in IntelliCage during a 15-day period. Dark period (07:00-19:00 h): **(A)** Total visits to operant corners. **(B)** Total nosepokes in operant corners. **(C)** Total water licks in operant corners. Light period (19:00-07:00 h): **(D)** Total visits to operant corners. **(E)** Total nosepokes in operant corners. **(F)** Total water licks in operant corners. Each data point is the overall number of occurrences per 12 hours calculated from the mean of 15 days per mouse. KCTD: K^+-channel tetramerization domain; WT: wild type. Data are presented as mean ± SEM (N=6 mice per genotype).